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Studies on the effect of toxin T-514 on the integrity of peroxisomes in methylotrophic yeasts

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1. SUMMARY

We have studied the response of two methylotrophic yeasts (*Hansenula polymorpha* and *Candida boidinii*) to toxin T-514, a toxin lethal to man, extracted from the shrub *Karwinska humboldtiana*. Growth experiments indicated a dose-response effect; at enhanced concentrations (50 μg/ml) the different subcellular organelles rapidly disintegrated resulting in death of the cultures. At non-lethal concentrations (< 2 μg/ml) growth ceased initially, but resumed after a lag period of 4 h. At the subcellular level a specific effect was observed on peroxisomal integrity. Distinct holes appeared in the peroxisomal membranes, resulting in leakage of matrix proteins from these organelles. In addition, import of newly synthesized proteins appeared to be blocked since cytosolic aggregates of matrix proteins were formed. The peroxisomal damage was probably irreversible since affected organelles were degraded at later stages of incubation. Upon restoration of growth on methanol, new peroxisomes developed from those which had escaped degradation.

2. INTRODUCTION

Karwinskia humboldtiana is a poisonous plant that grows in semi-arics of desert in north and central Mexico. It produces several compounds with different toxic effects [1]. One of these, termed T-514, causes severe damage to the lung, kidney and also the liver [2], where it induces the formation of large intracellular fat deposits and necrosis. Preliminary morphological observations suggested that one of the initial effects upon experimental poisoning of rats and monkeys includes a significant decrease of the number of microbodies (peroxisomes) present in hepatocytes

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(A. Piñeyro Lopez, unpublished results). In order to obtain more information on the specific effects of T-514 there may be on peroxisomes, we decided to explore the use of yeasts as model organisms in such studies. Yeasts seem to be favourable in this respect because i) they are easy to cultivate and ii) the proliferation and protein composition of peroxisomes in these organisms can be prescribed precisely by manipulation of the growth conditions [3–5].

As a first approach we studied the effects of different concentrations of toxin T-514 on the fate of microbodies in two methylotrophic yeasts, namely *Hansenula polymorpha* and *Candida boidinii*, grown at different levels of microbody induction. The results of these studies are reported in this paper.

3. MATERIALS AND METHODS

3.1. Microorganisms and growth

H. polymorpha de Morais et Maya CBS 4732 and *C. boidinii* ATCC 32195 were grown in batch cultures in mineral media [6,7] at 30°C and 37°C, respectively, on glucose (0.25%) or methanol (0.5%). T-514 was supplemented to the cultures at 0.5, 2, 10, 20 and 50 μg/ml, either (i) immediately after the shift to methanol or (ii) after the cultures had reached the mid-exponential growth phase on this compound. Samples were taken at 30 min and 1, 2, 4, 8 and 24 h after addition of T-514. The viability of T-514 treated cells was estimated after inoculation on YPD agar plates, using untreated cells as a control, (YPD: 1% yeast extract, 2% peptone, 2% glucose).

3.2. Biochemical analysis

Crude extracts were prepared as described previously [6]; protein was assayed as described in reference [8], alcohol oxidase as in reference [9] and catalase as in reference [10].

3.3. Ultrastructural analysis

$KMnO_4$-fixation [7] and immunocytochemistry using specific antibodies against alcohol oxidase and dihydroxyactone synthase and protein A/gold [11] were performed as already described.

4. RESULTS AND DISCUSSION

The growth experiments indicated that toxin T-514 had a drastic effect on the viability of both yeast species tested; independent of the composition of the growth medium, concentrations of 50 μg/ml were lethal and 100% of the cells were killed within 1 h of incubation. Light microscopical observations on methanol-grown cells of *C. boidinii* indicated an almost immediate effect on the overall subcellular organization after administration of the toxin. Within 10 min, most of the clusters of peroxisomes in the cells had migrated into the vacuole (Fig. 1A); subsequently, several large vacuoles developed, followed by the total disintegration of the cytosol. These observations were confirmed by electron microscopy. Especially the cellular membranes appeared to be affected (Fig. 1B) and after 2 h of incubation intact organelles could no longer be recognized. Comparable effects were observed in methanol-grown *H. polymorpha*. At all the concentrations of T-514 applied, distinct effects were evident (both physiologically and morphologically), but a clear-cut dose-response relationship was observed. The viability of both yeast species tested at different concentrations of the toxin are presented in Table 1. At the highest concentrations used (20 and 50 μg/ml) the cultures died quickly, showing overall cellular disorganization, as indicated above. However, at sub-lethal doses (0.5, 1 or 2 μg/ml) a specific effect was observed on the peroxisomal integrity of both strains tested. The

Table 1

Survival rates of cells of *Hansenula polymorpha*, grown in batch cultures on either 0.5% glucose or 0.5% methanol, after administration of various concentrations of T-514 in the mid-exponential growth phase (A_{660} approx. 2.0) (After 2 h of incubation cells were washed once with distilled water and inoculated on YPD agar plates. Data are presented as percentages of surviving cells, compared to untreated controls and represent the average of three independent experiments.)

Substrate	Concentration T-514 (μg/ml)					
	0.5	1.0	2.0	10	20	50
Glucose	> 90	> 90	70	20	5	0
Methanol	> 90	> 90	60	10	0–5	0

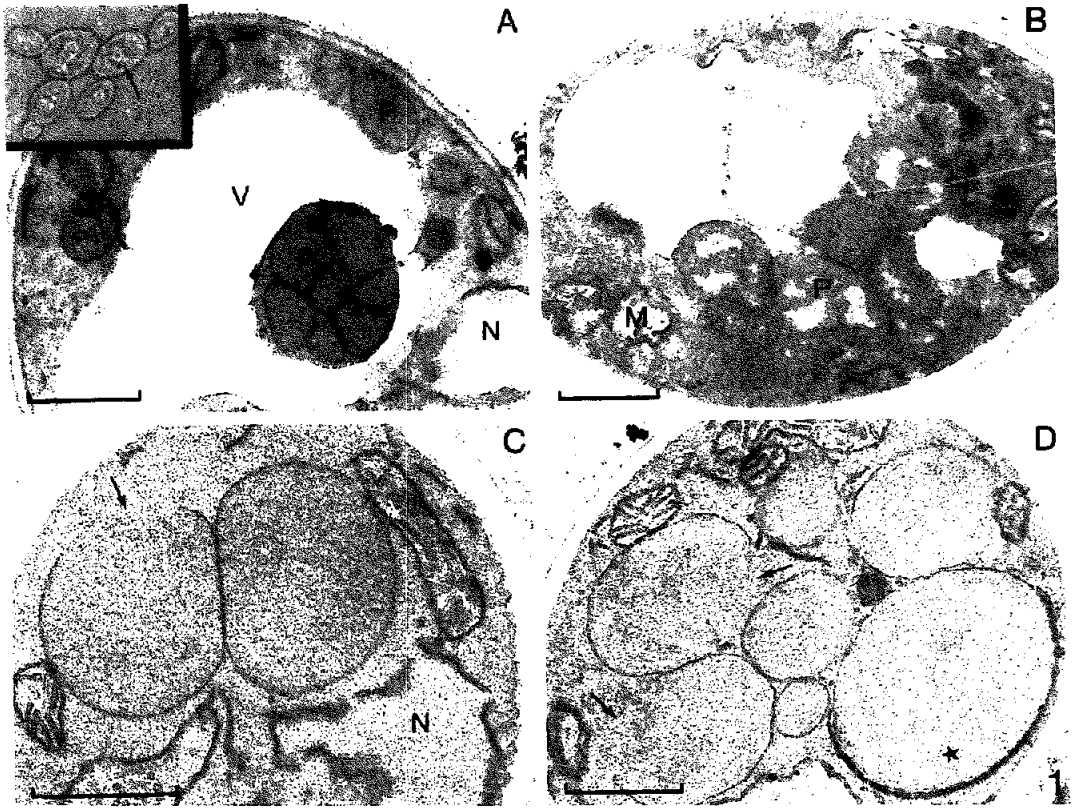

Fig. 1. **A** and **B** are of methanol-grown *C. boidinii*, supplemented with 50 μg/ml T-514. 10 min after administration of the toxin, clusters of peroxisomes have migrated into the central vacuole (**A**). Inset: light micrograph (2820×) of the same time point. After 30 min of incubation cell organelles are severely damaged (**B**); also cytosolic protein aggregates (*) are seen which are positively labelled after incubation of sections with anti-alcohol oxidase/protein A/gold (inset 2**B**). **C** and **D** are of methanol-grown *H. polymorpha*, incubated in the presence of 2 μg/ml T-514. After 30 min of incubation, distinct interruptions of the peroxisomal membrane (arrows) were observed. An initial stage of sequestration of a peroxisome to be degraded is also seen in **D** (*; 2 h of incubation with T-514). Abbreviations: A, autophagic vacuole; M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The marker represent 0.5 μm.

survival rates given in Table 1 for *H. polymorpha* did not differ markedly from the results obtained with *C. boidinii*.

In general, the following observations were made after addition of 2 μg/ml T-514 to batch cultures of each yeast in the exponential growth phase on methanol: a. growth ceased almost immediately; b. the peroxisomal membrane was affected; within 30 min of incubation, distinct holes were observed (Fig. 1C,D), resulting in the leakage of matrix proteins as was evident after immunocytochemistry (Fig. 2A). Damage to other cellular membranes (e.g., nuclei, mitochondria and vacuoles) was not detected under these conditions; c. import of newly-synthesized matrix protein apparently became blocked, judged by the appearance of large cytosolic aggregates of peroxisomal matrix proteins (alcohol oxidase and dihydroxyacetone synthase) within 30 min of incubation in the presence of the toxin (Figs. 1B,

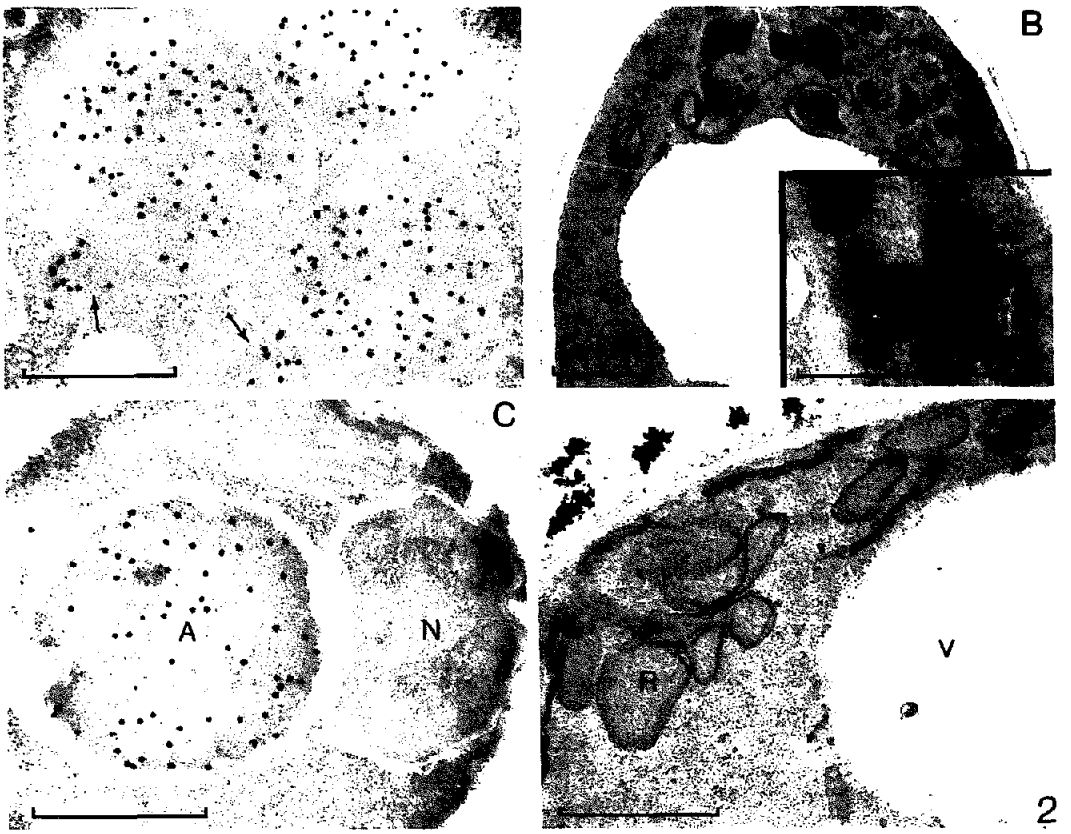

Fig. 2. **A** and **C**: immunocytochemical experiments on methanol-grown *H. polymorpha*, supplemented with 2 μg/ml T-514, using specific antibodies against alcohol oxidase and protein A/gold. After 30 min of incubation cytosolic alcohol oxidase is evident (**A**, arrow). **C** shows an autophagic vacuole, present in the cell after 4 h of incubation, containing alcohol oxidase protein. **B** and **D** are of methanol-grown *C. boidinii*, supplemented with 2 μg/ml T-514. **B** shows the presence of many cytosolic aggregates (*) after 2 h of incubation which are labelled after incubations with anti-alcohol oxidase/protein A/gold. Only few peroxisomes are left. **D** shows the initial stage of development of new peroxisomes upon restoration of growth on methanol, 4 h after administration of the toxin. Abbreviations and bar length as in Fig. 1.

2B); d. inactivation of peroxisomal matrix proteins was not observed; alcohol oxidase and catalase activities in both *H. polymorpha* and *C. boidinii* remained approximately constant in the first 2 h after administration of the toxin (data not shown); e. after 2–3 h of incubation, initial stages of the proteolytic turnover of individual damaged peroxisomes were observed (Figs. 1D, 2C).

Degradation occurred by the same mechanism as described previously for glucose-induced turnover of peroxisomes in methanol-grown cells of *H. polymorpha* [12]: peroxisomes to be degraded were first sequestered from the cytosol by

electron-dense membranes (Fig. 1D), followed by their uptake in the vacuole and degradation of the peroxisomal contents (Fig. 2C). After 4 h of incubation large autophagic vacuoles were frequently observed.

4 h after adminstration of T-514 at a final concentration of 2 μg/ml the cultures started to grow again. This process was associated with the development of a number of small microbodies, which grew rapidly in size during further incubation (Fig. 2D). The finding that proliferation of microbodies was not affected by the toxin was confirmed in experiments in which glucose-grown cells were shifted to fresh methanol-containing media supplemented with 2 μg/ml T-514. Although growth was initially abolished (as observed in methanol-grown cells) for 4–6 h, proliferation of microbodies initiated approximately 2–3 h after the shift of cells to methanol, occurred by mechanisms fully identical to those described for WT cells [4,7].

The proliferation effect in methanol-grown cells suggests that development of new peroxisomes was required to support continued growth on methanol. Hence, the damage to peroxisomes present before adding toxin, but which had escaped degradation, was probably not repaired. A comparable phenomenon was observed after placing KCN-treated methanol-grown cells of *H. polymorpha* into fresh methanol media [13]. As a result of the cyanide treatment, FAD was removed from octameric alcohol oxidase molecules, thus inactivating the enzyme without affecting the peroxisome integrity. Inactivation appeared to be irreversible; reappearance of alcohol oxidase activity depended on protein synthesis. This newly synthesized alcohol oxidase was, however, not imported into the inactive alcohol oxidase-containing peroxisomes originally present. Instead, the new oxidase was located in a number of newly developed small organelles. As also T-514 affected the peroxisomes containing inactive alcohol oxidase, and thus physiologically inactive, these peroxisomes were subject to proteolytic degradation upon further incubation of cyanide treated cells in fresh methanol media [13]. Sublethal doses of the toxin seem to have induced an impairment of specific peroxisomal functions, as growth on methanol is initially abolished, although the activities of peroxisomal key enzymes of methanol metabolism are not affected. As demonstrated in studies of peroxisome-deficient (*per*) mutants of *H. polymorpha*, growth on methanol is strictly dependent on the presence of intact peroxisomes [6]. Damaging the peroxisomal membrane may cause dissipation of the proton motive force across this membrane [14–16] and thus affect proper organellar function. Essentially, the leaky peroxisomes in T-514 treated cells are highly comparable to the large cytosolic crystalloids in *per* mutants of *H. polymorpha* which contain the bulk of the peroxisomal enzymes but lack the surrounding membrane. Detailed studies on these *per* mutants indicated that their inability to grow on methanol as sole carbon source was mainly related to the fact that, in the absence of an intact peroxisomal membrane, the cell has lost effective control of partitioning the fluxes of formaldehyde generated from methanol over dissimilatory and assimilatory pathways [6]. Administration of low doses of T-514 probably has a similar effect. However, the molecular mechanisms involved in the toxin/peroxisome interaction are still unknown and are a topic of current investigations.

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